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Oral reproductive and developmental toxicity of *Lignosus rhinocerotis* mycelium in rat

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ABSTRACT

Ethnopharmacological relevance: *Lignosus rhinocerotis* (*L. rhinocerotis*), also known as the tiger milk mushroom, is widely used as traditional medicine and as soup ingredient in Malaysia and Hong Kong. It is edible and is used traditionally for the treatment of fever, cough, asthma, wounds, chronic hepatitis, gastric ulcers and cancers. In view of its safety profile, little information is found in scientific literature. The objective of this study is to investigate developmental toxicity of *L. rhinocerotis* in pregnant Sprague-Dawley (SD) rats.

Materials and methods: Eighty pregnant SD female rats were used in this study for three treatment groups and a control group, each consisting of 20 pregnant female rats. Three doses of 850 mg/kg/day (Low-dose), 1700 mg/kg/day (Mid-dose) and 3400 mg/kg/day (High-dose) were selected for the study, whereas 10 mL/kg distilled water was served as the control. Examinations were conducted on pregnant rats and fetuses respects to mortality, body weight, body weights gains, food consumption and clinical observations. The pregnant females were gross necropsied on G20, followed by maternal and fetus examination, to evaluate the teratogenicity, reproductive and developmental performance of *L. rhinocerotis* mycelium.

Results: Results showed that no *L. rhinocerotis* mycelium-related animal death and abnormal clinical sign were noted. No statistical differences were noted in maternal mean body weight and maternal mean body weight gains. Some animals in the high-dose group appeared audible respiration due to dosing accident, it resulted in lower food consumption but not relevant to *L. rhinocerotis* mycelium treatment. In maternal gross necropsy, no *L. rhinocerotis* mycelium-related gross lesion was noted. In maternal examination, parameters of gravid uterus weight, implantation number, corpora lutea number, litter size, live or dead fetal number, male or female fetus number, resorption number, fetal sex ratio (M/F), pre-implantation loss and post-implantation loss were all within the normal reference ranges and showed no significant difference when compared to the control group. In fetus examination, including external, visceral and skeletal evaluations, there were no significant changes between any of the *L. rhinocerotis* mycelium treated groups and the control group.

Conclusions: Based on the study results, the no-observable-adverse-effect level (NOAEL) for pregnant female rats under the conditions of this study was 3400 mg/kg/day.

1. Introduction

Lignosus rhinocerotis (*L. rhinocerotis*) > (Cooke) Ryvarden, also known as the tiger milk mushroom, is a dimitic medicinal mushroom distributed in countries of China, Malaysia, Philippines, Sri Lanka, Australia and East Africa. It is edible and is used traditionally in Malaysia and Hong Kong to treat fever, cough, asthma, wounds, chronic hepatitis, gastric ulcers and cancers (Lee et al., 2009; Jones et al., 2007; Wong et al., 2009). According to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), *L. rhinocerotis* belongs to the family *Polyporaceae* and the order *Polyporales*. Its sclerotium appears as an irregular hard mass with no particular size. The outer skin of the sclerotium is rough and appears greyish brown in color. The inner section, however, has an off-white ivory granular texture with a slight milky odor (Yang and Fang, 2008). Pharmaceutical studies have indicated that *L. rhinocerotis* exhibits a variety of potential beneficial effects on human health, such as antiproliferation (Lai et al., 2008; Lee et al., 2012), immunomodulation (Wong et al., 2009). In addition, it also reported that *L. rhinocerotis* has

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antioxidative (Mohanarji et al., 2012), antimicrobial (Mohanarji et al., 2012), anti-viral (Kavithambigai et al., 2013), anti-coagulant (Sabaratnam et al., 2013) and neurite outgrowth stimulatory (Phan et al., 2013) effects.

Due to limited access of wild *L. rhinocerotis* in nature, the supply of wild *L. rhinocerotis* fails to meet the market demands. Artificially cultivated *L. rhinocerotis* is an ideal substitute in developing healthcare products. In our laboratory research, *L. rhinocerotis* mycelium was successfully isolated and identified from the fruiting body of *Lignosus rhinocerotis* which made mycelium culturing by fermentation possible. We yielded mass production of *L. rhinocerotis* mycelium using a variety of carbon source, nitrogen source and inorganic salts combined with liquid fermentation technique in a 50-ton fermenter, offering obvious advantages of less pollution and short production cycle.

Previous studies showed that no toxic effect were observed in mutagenicity and genotoxicity tests (Chen et al., 2013) and 90-day oral toxicity test (rats) (unpublished data). However, FDA requirements require substantial evidence of no harm for any article of commerce. Therefore, our aim of this study is to evaluate the adverse effects in the pregnant rats, embryofetal development and teratogenicity after administration of the test article, *L. rhinocerotis*, via oral gavage during the major embryonic organogenesis period (G6-G15). All results generated from this study will provide safety criteria information for human expose.

2. Materials and methods

2.1. Preparation of Lignosus rhinocerotis mycelium (Chen et al., 2013)

The test article used in the present study was freeze-dried *L. rhinocerotis* mycelium powder. *L. rhinocerotis* procured from Ligno Biotech Sdn. Bhd. (voucher code #0032) (Selangor, Malaysia) was grown on potato dextrose agar at 25 °C for 15 days, transferred to a 2.0-L flask containing 500 mL of nutrient medium of glucose 2%, soy bean powder 0.5%, peptone 0.2% and yeast extract 0.1% and $MgSO_4$ 0.01%, pH was adjusted to 4.0 with 0.1 M hydrochloric acid, and incubated at 25 °C on a rotary shaker at 100 rpm for 5 days. The fermented broth (500 mL) was inoculated into a 500L pilot seed fermentor containing 350 L of the same nutrient medium. The fermenter was cultured for 4 days before being transferred into a 20 t full-scale fermentor and cultured under the same conditions. The submerged mycelial culture was separated from the culture broth, lyophilized, then ground to powder and stored at − 20 °C.

2.2. Animals

A total of 180 male and female rats were used in this study. Male and female Sprague-Dawley CD® (SD) IGS rats (aged 9 weeks) were acquired from BioLASCO Taiwan Co. Ltd (Taipei, Taiwan). Animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility of Level Biotechnology Inc. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) before the beginning of this study (IACUC no. 120202-03). Rats were individually housed in polycarbonate cages excepting the cohabitation period with a 12 h light/dark cycle under controlled temperature (21 ± 2 °C) and relative humidity of 55 ± 20%. The animals had free access to standard rodent diet (Laboratory rodent diet 5010, LabDiet, PMI® Nutrition International, USA) and reverse osmosis water ad libitum from the day of reception until the end of the study. Each rat was identified by ear punch and was acclimated for 5 d after reception. Rats were examined daily for any physical abnormalities and weighed prior to the experiment.

Table 1
Maternal mortality/moriboundity.

Group	Dose (mg/kg/day)	Mortality[a]	Effective animal number
Vehicle control	0	0/25	25
Low-dose	850	0/26	26
Mid-dose	1700	0/23	23
High-dose[b]	3400	1/23[b]	22

[a] N/N: Number of rats with death or moribound/Pregnant animal sample number.
[b] One animal (ID A120500250056) was found dead at G14 due to dosing accident, it was not test article related.

2.3. Mating

For mating, animals were randomized mated by placing one female in a cage of one male. Evidence of mating was determined by the presence of vaginal sperm and/or vaginal copulation plug. Females considered to have successfully mated were removed from the cage of the male, weighted and assigned a gestation day (G0), and housed individually. Female rats remained in cohabitation with the second set of males until either the desired number of mated females was reached (N ≧ 20 per group). Once the required number of mated females reached, all males and the remaining females were excluded from the study. All animals excluded from study were euthanized by carbon dioxide inhalation without any further observation.

2.4. Study design

This study was performed based on the Safety Evaluation Methods for Health Food (1999). DOH, Taiwan, R.O.C. and was conducted in accordance with Good Laboratory Practices (GLP). To prevent the adverse effect of oral gavage, the volume of *L. rhinocerotis* mycelium administered was calculated as 10 mL/kg. *L. rhinocerotis* was prepared freshly every day and administered to each rat. The test animals were not fasted before gavage. Animals were administered *L. rhinocerotis* mycelium at dosage levels of 0, 850 (low), 1700 (medium), and 3400 (high) mg/kg body weight during the major embryonic organogenesis period (G6-G15). Copulated dams were observed once daily for any clinical signs of toxicity and death. Body weights and food consumption were verified and recorded on G0, 3, 6, 9, 12, 15, 18, and 20.

On gestation day 20, animals were euthanized by carbon dioxide inhalation followed by exsanguination and immediately subjected to a laparohysterectomy. The number of corpora lutea in each ovary was recorded after the uterus was removed and weighed. The number of early and late resorptions, viable and nonviable fetuses, fetal sex, and the number of implantation sites were recorded. Each fetus was individually weighed and examined for external abnormalities. For visceral examinations, fifty percent of fetuses of each litter were blotted dry and fixed in Bouin's solution for subsequent gross examination. Each fetus was examined using Wilson's razor-blade sectioning technique for any internal organ anomalies. For skeletal examinations, fifty percent of fetuses of each litter were euthanized, eviscerated, and fixed in 95% ethyl alcohol. After fixation, fetuses were submersed in aqueous potassium hydroxide, stained with Alizarin Red S and Alcian Blue, and cleared in glycerin for subsequent skeletal examination. The skeletons of each fetus were examined for completeness of bone ossification and any malformations or variations in the skeleton. All findings were recorded.

2.5. Statistical analysis

All measured parameters were calculated and expressed as mean ± standard deviation or percentage. The litter is the experimental unit for evaluation where appropriate. Comparisons of data (maternal body weight, maternal body weight gains, food consumption, gravid uterus weight, fetal weight/length, implantation number, corpora lueta num-